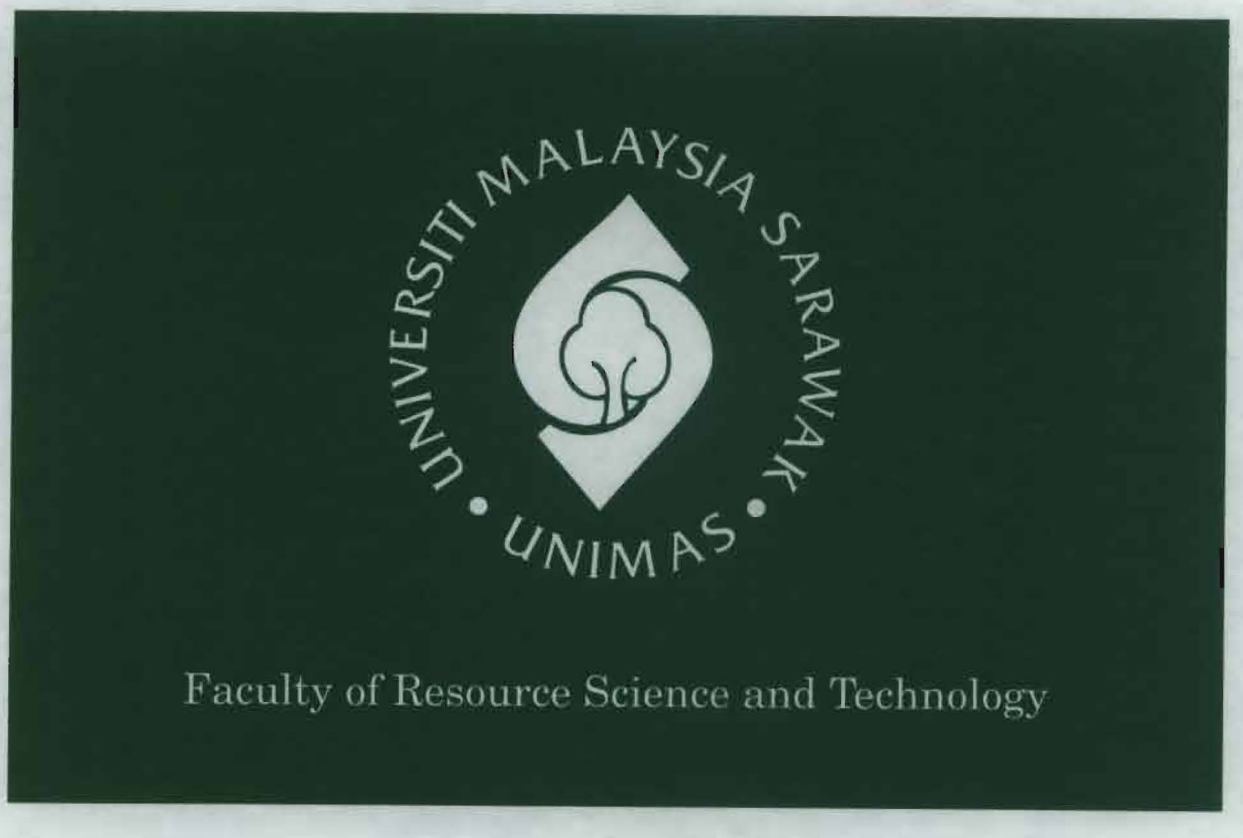

ISOLATION OF CDNA FRAGMENT ENCODING STARCH SYNTHASE GENE FROM *Metroxylon sagu* BY RT-PCR METHOD

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Bachelor of Science with Honours
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This report is submitted in partial fulfillment of requirement for the degree of Bachelor of Science with Honors (Resource Biotechnology)

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Abstract

Metroxylon sagu have a big potential to compete with wheat, barley, rice and corn as food supply for human population in this region. Our understanding of starch biosynthesis and chemistry in most of important food supplier plant has advanced rapidly over the last few years, but our knowledge for starch synthesis in *Metroxylon sagu* is still lacking. In order to under stand the biochemistry of starch in *Metroxylon sagu,* genes that control starch biosynthesis in sago palm have to be known first. Degenerated primer for targeting a specific gene of starch synthase in *Metroxylon sagu* have been design using other plant starch synthase sequences that are closely related to *Metroxylon sagu.* It is found that this primer could amplify targeted gene at temperature ranging between 40°C to 48°C, but this primer does not amplified the full length of starch synthase gene. An expected fragment targeted by this primer was 1.2 kb in length but the cDNA fragment that was isolated has a size of 100 bp to 200 bp in length.

Keyword: degenerated primer, cDNA, starch synthase, *Metroxylon sagu*

Abstrak

Metroxylon sagu mempunyai potensi yang besar untuk bersaing dengan gandum, barli, padi dan jagung sebagai sumber makanan utama manusia. Bagi kebanyakan daripada tumbuhan ruji ini telah diketahui biositesis dan komposisi kimia kanjinya beberapa tahun yang lalu tetapi malangnya kita masih tidak mengetahui hal ini bagi pokok sagu. Bagi memastikan kita memahami bagaimana kanji pokok sagu terhasil, gen spesifik yang mengawal proses ini perlu kita kenal. Degenerated primer khas untuk mengenal gen starch synthase dalam pokok sagu telah dibuat berdasarkan jujukan gen yang sama dalam beberapa tumbuhan yang hampir dengan pokok sagu. Didapati bahawa primer yang telah dihasilkan boleh memperbanyakkan gen tersebut pada suhu 40°C hingga 48°C tetapi ia cuma sebahagian dari gen tersebut. Degenerated primer ini dijangkakan akan menghasilkan 1.2 kb fragmen gen daripada keseluruhan starch synthase gen tetapi hasil menunjukkan fragmen yang panjangnya 100 bp ke 200 bp.

Kata Kunci: degenerated primer, cDNA, starch synthase, Metroxylon sagu

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Chapter 1

Introduction

Wheat, barley, corn and rice are the important sources of food for us to live but it is not enough to supply the whole human population that increases day by day. According to Mc. Clacthey *et al.* (2004), *Metroxylon sagu* (*M. sagu*) had been found to be the highest starch producing plant. If we are able to study how starch in *M. sagu* is made, then we are able to manipulate it and make it become one of the food supply plant for over the world. This study was conducted in order to isolated cDNA fragment of starch synthase gene from *M. sagu* and for that reason, a specific primer has been designed to detect specific sites of the target gene from total RNA that have been isolated. That is why a specific primer is needed in order to detect the target gene from the total RNA that has been isolated.

Studying gene expression requires mRNA extraction and formation of more stable molecule known as complementary DNA (cDNA). cDNA is a genetic material that is produced from mRNA. It was done by using reverse transcriptase enzyme that only can be found in retroviruses. cDNA was chosen because it does not have any 'junk'

gene or intron that does not encode any protein. By doing this, specific gene for specific protein or enzyme can be obtained. Sago palm consider to have high potential of becoming one of the most important food supply, in a sense that these studies will only focus on enzyme that involve in starch production. Yet, the result of this project is just a fragment of starch synthase gene that have been isolated and amplified, because of partial degenerated primer was used. Future aims of this study are to characterize this gene and clone it.

Plants synthesize carbohydrate via photosynthesis and store it in the form of starch. Starch is the most significant form of carbon reserve in higher plants in terms of the amount it made (Smith and Martin, 1995). According to Hedley *et al.* (1997), starch composes of two distinct polymers that are amylose and amylopectin. Badenhuizen (1969), and Preiss (1988), reveal that the basic starch biosynthesis is similar in all plant tissue. It consists of three main events in the chloroplast and amyloplast (Muller-Rober and Kossmann, 1994): (a) supplying the Glucose-1-Phosphate into plastid; (b) synthesis of ADP-Glucose; and (c) synthesis of starch. As in diagram 1, the biosynthesis in plastid involve a series of enzyme including ADP-Glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (SBE) and starch debranching enzyme (SDBE).

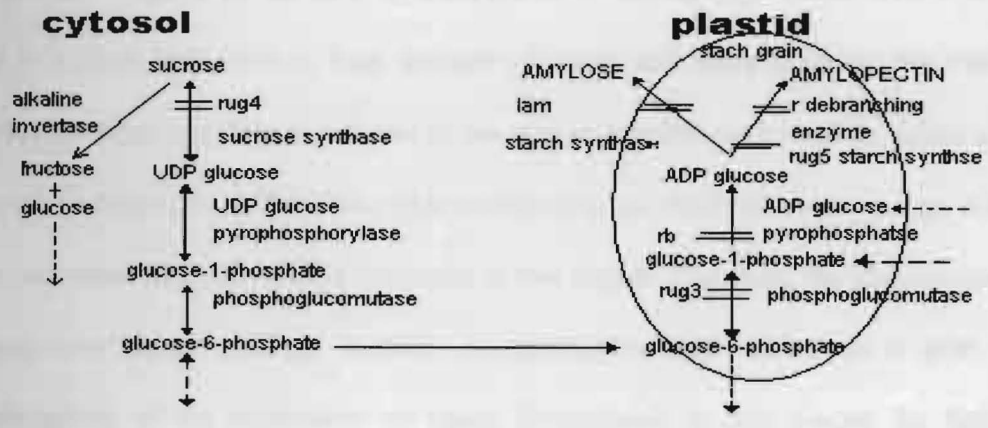

Diagram 1; Starch biosynthesis pathway in pea. (Adapted from Wang *et al.*, 1997)

Normal ratio for amylose: amylopectin in plant is 30: 70, (Smith and Martin, 1995). However, starch characteristic in granule shape and amylose: amylopectin ratio, is differ among tissue in the same plant. According to Sato (1984), this is because of the different of enzyme isoform and the process in different types of tissue. In order to understand properly each step of starch production in certain species, we need to characterize the gene. The aim is simple, that is to manipulate the process for human benefit which also can overcome nutritional problems that occurs all over the world.

Today, human populations have increase greatly than before. Lots of problems especially in food occur everywhere. Foods are always not enough for every single one of us. For that sense, we need to find other alternative that can support our food

supply in the future. According to Nakamura *et al.* (1998), sago palm is known as one of the oldest tropical plants used by mankind in producing sago starch. *Metroxylon sagu* is a plant that produce high amount of starch and store it inside the trunk. *Metroxylon sagu* is widely distributed in the area of east-Malaysia such as Sabah and Sarawak (Anonymous, 2004). This wide distribution had made *Metroxylon sagu* itself as an important material of food industries in this region. Therefore, the identification of particular starch synthase isoform in *Metroxylon sagu* will open a goal in understanding of the mechanism of starch biosynthesis in this species for future information use.

The main objective of this research is to detect the target gene that is the starch synthase, using degenerated primer that have been designed and amplified it by using RT-PCR method. Our goals in archiving this objectives is: a) to gain total RNA from *Metroxylon sagu* leaf tissue samples, b) able to synthesize cDNA, c) able to synthesize first strand and second strand using degenerated primer, and d) amplified the gene using gradient PCR machine. The hypothesis of this study was, only a partial of starch synthase gene was amplified and targeted. It has been assumed that the degenerated primers that have been design were able to targeted specific site on starch synthase gene.

Chapter 2

Literature review

Metroxylon sagu (sago palm)

M. sagu locally known as sagu or rumbia tree to local people grows well in surrounding of swampy area. It is a flowering plant that only blooms once in their life time. Its trunk may reach up to 10 m to 14 m height and 30 cm to 60 cm in diameter (Saidin, 1993). The trunk of sagu contain starch that act as a food reservoir for flowering and fruiting (Flach, 1996). Each trunk can yield about 250 lbs to 650 lbs of starch in the form of sago pearl (Anonymous, 2004).

Mc. Clatchey *et al.* (2004), found that *M. sagu* only can be found in the area around Papua New Guinea, Indonesia and Malaysia. This gives a reason why sago palm potential hasn't been reveal and we are yet to discover how the genes of starch synthesis in sago palm work.

According to Danjaji *et al.* (2001), major cultivation of sago tree in Malaysia is in Sarawak. It reaches up to 25 tonne of starch per hectares. The ratio of starch yield

towards other crops like cassava and maize are 2000 – 3000 kg/Ha per Yr. whereby 1000 kg/Ha per Yr. in maize (Stantan, 1992) and 2000 kg/Ha Yr. in cassava (Ahmad *et al.*, 1999 cited in Barozah, 2004). Mohayidin *et al.* (1998) as cited in Barozah (2004), reveal that Sarawak is the world biggest sago exporter in the word where it reach 25 000 tones to 40 000 tones per year.

Sago starch can be a very important material in much kind of food industries such as manufactured of sodium glutamate, glucose, high fructose syrup, noodles, caramel, cracker, sago pearl, stabilizers and texture risers (Jahan, 2004). Bujang *et al.* (2000), mention that sago starch is also an important material in making paper, textile, plastic, alcohols, adhesives and paper glue. According to Mc Clatchey *et al.* (2004), sago palm will be become one of starch sources for all over the world because the trunk can produce such a big amount of starch. It is enough for industries and food supply for all being that depend on starch for life.

Starch biosynthesis

Starch biosynthesis involves a series of enzyme activity. Diagram 1 show the starch biosynthesis pathway. There are three main enzymes that are referred to, ADP-Glucose pyrophosphorylase (AGPase), starch synthase (SS) and starch branching enzymes (SBE). AGPase are responsible for the synthesis of substrate for starch biosynthesis in all plant tissues (Smith *et al.*, 1997). Supplies of ADP-glucose by AGPase is the most significant factors in determine the rate of starch accumulation.

Lin *et al.* (1988) proved this fact by referring to experiment that shows there are only 7% of starch produce in mutant leave compare to 26% of starch produce in normal leaves. Preiss and Sivak (1996), also have the same idea of AGPase.

AGPase is sensitive to allosteric regulation by 3-Phosphoglyseraldehide (3-PGA) and free phosphate (Pi) which able to increase starch biosynthesis in cultured tobacco cells by 30% and in potato up to 60% by transformation in *Escherichia coli* gene but other allosteric regulation cannot increase or decrease starch production (Smith and Martin, 1995). The degree of regulation exercise by 3-PGA and Pi could be different between the isoform of AGPase. According to Muller-Rober and Kossmann (1994), mention that modification of enzyme leads to decrease or increase of starch content or product in starch biosynthesis.

Starch synthase will extend α-(1-4) glucan chain and synthesize both amylose and amylopectin. There are five subfamilies of starch synthase, granule bound starch synthase (GBSS) and starch synthase I, II, III and IV (Dian *et al*, 2005). Sprage *et al.* (1943), discovered that waxy mutants of maize, rice, sorghum and *amaranthus* along with amylose free (amf) mutant of potato, makes no amylose and lack the activity of an exclusively GBSS. This also proven by Jacobsen *et al*, (1989) that cited in Smith and Martin (1995), which referred it as waxy protein experiment. GBSS is attached to the starch granule while other isoform of starch synthase are located in the soluble phase of the amyloplast, but there are some located in both phase (Smith, 1990).

The members of subfamilies that certainly active in different parts of the plant and in different species are relative to contribution of synthesizing different α-(1-4)glucan (Denyer *et al.*, 1995). Soluble and granule-activities present in all tissue, but whether an enzyme is exclusively or partly associated with starch granule appear to be intrinsic property of the protein rather than starch. This indicates by experiment using transgenic by Edward *et al*, (1996) and Smith *et al.* (1997). GBSS I believe to be responsible for the synthesis of amylose (Smith and Martin, 1995; Preiss and Sivak, 1996).

SBE are responsible for creating the α-1,6-linkage of amylopectin (Wang *et al.*, 1998). According to Burton *et al.* (1995), enzymes from a number of different plant fall into two different classes based on their primary sequences. Generally, in storage tissue such as cotyledon or endosperm, hexose phosphates are import into the amyloplast where as fructose-6-phosphate from the reductive pentose phosphate pathway is utilized in photosynthetic plastid.

Primer Design

According to Innis and Gelfand (1991), primer that will be design should be in range of 17 – 28 based length. Based composition should be 50 – 60 % contains of GC and the estimated temperature should be in the range between 55 – 80°C. This is to ensure the primers that have been designed are able to attach and amplified sequence at

maximum temperature given. Most oligonucleotide synthesis reactions are only 98% efficient (Innis and Gelfand, 1991).

Yap and Mc Gree (1991) said that Taq-polymerase is given as having a half life of 30 minutes at 95°C, so the cycle for PCR should not be exceeding 30 cycles. But it is possible that the denaturing temperature to be lowed but the length of sequence targeted also decrease. They also found that a pair of primer that has a very different annealing temperature may never give appreciable yield of a unique product.

Annealing temperature of PCR depends directly on the length and composition of the primer, so when designing a primer annealing temperature should be aim about 5°C below the lowest estimated temperature for the pair of primers that is design (Innis and Gelfand, 1990 cited in Coyne *et al.*, 2001). Thompson (2000) found that any automated sequencing product has a finite probability of producing error. The primer that was designed using these types of sequence is shifted too far from the exact sequence of the gene. The primer consideration is that, the primer should be complex enough to prevent the likelihood of annealing to sequence other than the chosen target. Primers pair sequences should also design to have lower chances of having hybrid among them.

cDNA synthesis

cDNA synthesis use mRNA strand as templates (Brazma *et al.*, 2001). Total RNA that extract from sources gives problem where all types of RNA are there (Strachan and Read, 1999). The mRNA has unique characteristic where it has poly (A) + tail that is compliment with oligo-dT chain (Russell, 1992). This characteristic can be applied to isolates the mRNA from total RNA. Reverse transcription (RT) enzyme can be use to synthesize cDNA because of its ability to reverse the transcription process of DNA polymerase to form cDNA from mRNA templates. There are two types of RT enzyme, (1) avian RT enzyme; and (2) murine RT enzyme (Sambrook *et al.*., 1989). Oligo-dT chains act as primer for RT enzyme reaction to make cDNA strand copy of mRNA templates (Russell, 1992).

In cDNA synthesis activity, there are also other enzymes that involves which are; a) RNase H to cut the mRNA strand to form second strand of cDNA, b) DNA polymerase to makes second cDNA strand and c) DNA ligase to ligate the strand (Okayama and Berg, 1982). According to Sambrook *et al.* (1989), there are three major qualities to be determined, (1) the efficiency; (2) the product of the first-strand reaction can be used directly without further treatment and purification, and (3) it does not involve S1 nucleases enzyme in order to cleave the single-stranded hairpin loop.

Reverse transcription-polymerase chain reaction (RT- PCR)

RT-PCR is the most sensitive technique used to detect mRNA and the quantizations is available (Anonymous, 2004). It can be used to quantify much smaller quantities of samples. RT-PCR is actually a modification of basic PCR that used thermostable DNA polymerases. In order to study the RNA using basic PCR, it must be reverse transcribed into cDNA using RT enzymes to provide necessary DNA templates for the thermostable polymerases. This process is called reverse transcription where the RT-PCR name came about.

Generally, avian myeloblastosis virus (AMV) or molonery murine leukemia virus (M-MLV or MuLV) reverse transcriptase are used to produce DNA copy from RNA templates using either random primer, oligo (dT) primer or a sequence-specific primer (Pozo *et al.*, 1998). The basic PCR reaction is carried out after the initial reverse transcription step has produced the cDNA templates. The starting RNA templates can be used total RNA or poly (A) + tail RNA. This research conducted using the total RNA as templates for starting the RT-PCR. RevertAid^{TM} H minus M-MuLV Reverse Transcriptase that used in the study is genetically modified M-MuLV RT. It differs from the M-MuLV RT by its structural on catalytic properties. The enzyme that is used posses RNA- and DNA dependent polymerase activity but lacks of ribonucleases H activity specific to RNA in RNA-DNA hybrids. RNase H activity is eliminated by a point mutation in RNase H domain of M-MuLV RT. (Anonymous, 2003)

Chapter 3

Methodology

Degenerated Primer

Multiple sequence alignment ClustalW (v.1.82) program was used to gain conserve region of starch synthase protein sequences that were taken from NCBI databases. Both protein and mRNA sequences of starch synthase that have been used for designing primer were *Tricum aestivum* GBSS, AY050174; *Tricum aestivum* SS I, AF091803; *Oryza sativa* SS II, NM 196673; and *Zea mays* SS II, AF019296. Conserve sequences region were assigned based on the presence of at least one variant residues in the 28 polypeptides analyzed and conservative substitutions of several nearby residues in the same positions in the alignment among all of the sequences (Cao *et al.,* 1999).

According to Thompson (2000), when designing a working primer, there are certain aspect to considered such as; a) the accuracy of the sequence that were used, b) the search region must reflex the goal, c) the sequence for the primer should be locate correctly, d) discard any sequences that shows undesirable self-hybridization, e) site

specific primer should be verified and sequence that was chosen should be rich in A-T at the 3' end.

Estimated melting temperature (Tm) estimated as Tm = [4(G+C) + 2(A+T)] ° C - 5° C. Estimated melting temperatures for both reverse and forward primer are 62° C. The length of primers that have been designed ware 18 bp. All homologous sites were considered to be taken as primer. Before primers sequences were generated to be working primers, it has to be simulated and tested using SeqTools software to check the performance of primers on simulated PCR test. This step also considered to be isolations of sequences method. The results that arise from this method have been compared in order to select the best primer sequences among all generated primers that have been taken out from homolog sequences. All of these were only done by using single software called SeqTools.

Estimated melting temperature (Tm) of the best sequence result from simulation and isolation were calculated. It was considered to be a forward primer. Second sequence from the best sequences was then reversed to gain reverse primer. The estimated annealing temperature for reverse primer sequences was calculated. Any pair primers sequences that were having estimated temperatures in very different range were eliminated. The best pair sequence that gives nearer estimated annealing temperature was taken to be as working primers.

Sequences of primers were then sent to 1st Base Company for generating working primers that were used in this research. Degenerated primers that were used as working primers have estimated temperature of 55°C for both forward and reverse primer. Annealing temperature for these primers should be around 50°C. Pellet primers that have been received back from 1st Base company were diluted in TE buffer to get 100 pmol concentrations of primers in a solution. Primers solution was ready to be used for lab work and then stored in 4°C. Result of multiple alignments is available in appendix A and primers sequence that have been design is available in appendix B.

PCR with cDNA Library

PCR machine that were used was Gradient PCR. It has the ability to run numbers of different temperature in one run. This method was modified from method that given by Fermentas in Taq-Polymerase for PCR kit. Standard PCR condition was used for amplifying the gene that was targeted: 1X PCR buffer, 1.5 mM $MgCa^{2+}$, 0.2 mM dNTP's, 1 µM primer (reverse and forward), and 1 U Taq polymerase. Sequence of primers that was used for this experiment is available in appendix B. Template DNA samples were taken from cDNA library from leaf tissue of *Metroxylon sagu* that was construct by Barozah (2004). PCR solutions were prepared as in table I.

Table 1; PCR reaction mixtures

Material	Stock solution	Aliquots	Working solution	Aliquots	PCR solution
dH_2O	-	-	-	Add 24µl	
PCR Buffer	10X (1.5 ml)	direct	200 µl	5 µl	1X
dNTP's	2 mM (1 ml)	direct	200 µl	5 µl	0.2 mM
Primer 1(F)	100µM (422µl)	50µl	25µM (200 µl)	2 µl	1 µM
Primer 2(R)	100µM (390µl)	50µl	25µM (200 µl)	2 µl	1 µM
Taq	5 U/µl (1 ml)	20µl	0.5U/µl(200µl)	4 µl	1 U/µM
Polymerase	25 mM (1 ml)	Direct	200 µl	3 µl	1.5 mM
$MgCl_2$	-	-		5 µl	
Templates			200µl each	50 µl	
Total					

PCR is very sensitive to contamination. To avoid any contamination, some step have been taken; a) all reaction mixture, DNA sample preparation and PCR process have been performed in separate area, b) laminar flow cabinet with UV light was used for preparing the reaction mixture, c) fresh gloves was worn for DNA purification and each reaction set up, d) reagents were prepared separately, and e) all solutions were autoclaved except for dNTP's, primers and Taq-Polymerase. Parameters for PCR reaction was set as below.

Initial denaturation	94°C	5 minutes	
	94°C	1 minute	25 cycles
	Annealing, 1 minute		
	72°C	2.5 minutes	
Final extension	72°C	7 minutes	

*annealing temperature: 55°C, 54°C, 53°C, 52°C and 51°C

Total RNA Extraction

This method was taken from Hussain (2002) cited in Barozah (2004). 1.5 g to 2.0 g of frozen leaf sample was grounded using motar and pestel until it became powdered. 15 ml of RNA extraction buffer (150 mM LiCl, 50 mM Tris pH 8.0, 5 mM EDTA, and 5% w/v SDS) was added to the sample and was vortex. 15 ml of phenol/chloroform/isoamyl (25:24:1) was added to the solution and then been vortex again, follow by centrifugation at 8,000 rpm for 1 minutes in 4°C.

Then the solution was washed using 15 ml of phenol/chloroform/isoamyl (25:24:1). It was performed three times followed by three extractions with 15 ml of chloroform to the sample and it had been vortexes and spin at 8000 rpm for 1 minutes in 4°C. The result was aqueous solution containing RNA after precipitation by adding 8 M LiCl that was giving a final concentration of 2 M LiCl for RNA precipitation. All pellet and solid phase from centrifugation was discarded.

The extract was then incubated for overnight at 4° C and then it then was centrifuged at 8,000rpm for 25 minutes. RNA pellet was suspended in 400 µl of sterile distils water. RNA was precipitated again in 10 µl of 100% (v/v) ethanol and 40 µl 3 M Na acetate and had been put on ice for 20 minutes. Then it was centrifuged at 13,000 rpm for 25 minutes followed by washing twice with 70% ethanol. The palette was dried in air and had been suspended in 100 µl of TE buffer (10 mM tris-HCL ph 8.0, 1 mM

EDTA) store at -70° C. This solution was then store at -20 °C and ready for use. 5µl of sample have been visualized using electrophoresis gel in TBE buffer.

Treatment with DNase 1

Before Total RNA sample that have been extracted were used for synthesizing first strand cDNA, it was treated using DNase free-RNase kit (fermentas, catalog no. # M6101). Digestion reactions were ready as in table 2.

Table 2; DNase free-RNase digestion reaction.

RNA in TE buffer	1-8 µl	(5 µl)
RQ1 RNase-Free DNase	10X Reaction Buffer	(1 µl)
RQ1 RNase-Free DNase	1 U/µg RNA	(1 µl)
Nucleases-Free water to a final volume of 10 µl		(3 µl)
Total		(10 µl)

When reaction solution was ready, it is then incubated at 37°C for 30 minutes and then the reaction was stopped by adding 1 µl of RQ1 DNase solution. It was incubated at 65°C for 10 minutes to in activate the DNase reaction. This sample was then directly used in first strand synthesis.

First Strand Synthesis

Synthesizing first strand cDNA from total RNA was using the reverse transcriptase enzyme. Sample that has been treated using DNase treatment was directly used in synthesis of first strand cDNA. Procedure that has been used was manual step that

provide together with Fermentas kit. Reaction mixture was prepared in a tube on ice as in table 3.

Table 3; First strand synthesis reaction mixtures.

Total RNA 10ng – 5ng	(5 µl)
Sequence specific (15-20pmol)	(1 µl)
DEPC-treated water to 12 µl	(6 µl)
Total	(12 µl)

The solution was then mixed gently and had been centrifuge briefly. Then it was incubated at 70°C for 5 minutes before it chill on ice and been centrifuged again. Then another reaction solution was added to the mixture as in table 4.

Table 4; Reaction solution for first strand synthesis.

5X reaction buffer	(4 µl)
Ribolock™ Ribonuclease inhibitor 20U/µl	(1 µl)
10mM dNTP mix	(2 µl)
Total	(19 µl)

Then this solution was gently mixed and had been centrifuged. It was then incubated again at 37°C for 5 minutes and had been added with RevertAid™ H minus M-MuLV RT 200 U/µl (1 µl). Final volume for this reaction was 20 µl. After that it had been incubated for 60 minutes at 42°C and the reaction was stop by heating it at 70°C for 10 minutes. Finally, it was chilled on ice immediately before it was stored at minus 20°C. 5 µl of sample has been used for visualization with electrophoresis gel in TBE buffer.

Treatment RNase H

Product from the first strand synthesis was divided into two samples and one of it was treated with RNase H to have pure first strand sample. The enzyme kit that was used is RevertAid H minus M-MuLV Reverse Transcriptase from Fermentas. This enzyme specially degrades only RNA strand in RNA-DNA hybrids. It does not hydrolyze phosphodiester bond within single-stranded and double-stranded DNA or RNA. This method was taken from protocol that provide along with this kit. Reaction solution has been prepared as in table 5.

Table 5; reaction solution for RNase H

RNA (1st strand) template	10 µl
RNase H buffer	2.5 µl
RNase H enzyme	1.0 µl
DEPC water	11.5 µl
Total	25 µl

Reaction solution was then incubated at 37°C for 40 minutes. After 40 minutes, the mixture was incubated again for 30 minutes at 65°C to denature the RNase H. 5 µl of sample was taken out for visualization in agarose gel electrophoresis. The sample was then stored at -20°C.

Second Strand Synthesis

This method was taken directly from the method that has been included together with fermentas kit for second strand synthesis. Both samples on first strand cDNA were diluted to 0.25ng. The concentration of first strand was quantitated using